Molecular Pathogenesis of Genetic and Inherited Diseases

Lithium Reduces Tau Phosphorylation but Not Aβ or Working Memory Deficits in a Transgenic Model with Both Plaques and Tangles

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Glycogen synthase kinase 3 (GSK-3) is a major kinase implicated in the pathogenesis of Alzheimer's disease (AD), and reducing its activity may have therapeutic efficacy. Two variants exist, referred to as GSK-3α and GSK-3β. In addition to the latter's well-described role in the phosphorylation of tau, reports also suggest that GSK-3α may regulate amyloid precursor protein processing and Aβ formation. The activities of both GSK-3α and GSK-3β are reduced by lithium, a well-tolerated drug used in humans to combat bipolar disorder. Here, we investigate the therapeutic efficacy of chronic lithium administration in aged 3xTg-AD mice that harbor both plaques and tangles. We found that lithium reduced tau phosphorylation but did not significantly alter the Aβ load. Despite the reduction in phosphotau, lithium treatment did not improve deficits in working memory. Although other studies have investigated the effects of lithium on tau biochemistry, this study represents the first to address comprehensively its therapeutic potential on other critical aspects of AD including its effect on Aβ and learning and memory. It remains to be determined from human clinical trials whether lithium treatment alone will improve the clinical outcome in AD patients. These results, however, suggest that the most efficacious treatment will be combining lithium with other anti-Aβ interventions. *(Am J Pathol 2007, 170:1669–1675; DOI: 10.2353/ajpath.2007.061178)*

Alzheimer's disease (AD) is characterized by a progressive deterioration in cognitive function, including a profound loss of memory. Accumulation of amyloid plaques and neurofibrillary tangles represent the two neuropathological hallmark features of AD,[1] which are formed by accumulation of the small peptide amyloid-β (Aβ) and the microtubule-binding protein tau, respectively. These lesions mainly accumulate in brain regions known to modulate cognitive functions, such as the hippocampus, cortex, and amygdala. Many AD-directed therapies are aimed at lowering the Aβ burden, whereas fewer anti-tau therapies have been evaluated in either mouse models or in human clinical trials. One potentially feasible approach at lowering the tau burden involves reducing the activity of the kinases that phosphorylate tau. Several kinases have been implicated as key players involved in tau hyperphosphorylation, particularly glycogen synthase kinase 3β (GSK-3β) and cyclin-dependent kinase-5 (CDK5).[2,3]

Lithium is a potent inhibitor of GSK-3 and is commonly used for the treatment of bipolar disorder and seems to be well tolerated in humans.[4] Given the critical role that GSK-3 plays in the pathogenesis of AD,[3] it represents an important drug target. Conflicting reports exist as to the efficacy of lithium on tau phosphorylation *in vivo*. Noble and colleagues[5] showed that chronic lithium administration in a transgenic mouse model overexpressing tau_{P301L} reduces tau phosphorylation at specific sites targeted by GSK-3β. In contrast, lithium had no effect on tau phosphorylation in another transgenic model overexpressing human wild-type tau.[6] In addition, there are reports that GSK-3α may affect amyloid precursor protein (APP) processing, suggesting that lithium may also lower Aβ levels in the central nervous system.[7] Again, there is some controversy because *in vitro* reports show that lithium increases Aβ levels, whereas a few *in vivo* studies indicate that lithium reduces Aβ levels in transgenic mice overexpressing human APP.[7–9]

Lithium holds great promise as a potential therapy for treating AD, particularly because it may affect both

pathological hallmarks of AD, and currently there is a clinical trial testing the effects of lithium on AD patients (Leon Thal, AD Cooperative Group, University of California, San Diego, CA). Several critical questions, however, have not yet been addressed, including whether lithium will be able to reduce tau hyperphosphorylation in a model with concomitant plaque pathology and whether it can rescue the associated cognitive impairments. Here, we evaluated the effects of chronic lithium in the 3xTg-AD mouse model, which develops plaques and tangles, as well as soluble $A\beta$ oligomers, in an age- and region-dependent manner.[10–12] We found that lithium reduced selective phosphotau sites, particularly those targeted by GSK-3β, but had no effect on the $A\beta$ load and was unable to restore working memory in this mouse model.

Materials and Methods

Mice

The derivation of the 3xTg-AD mice used in this study has already been described.[10,11] In brief, the 3xTg-AD mice were derived by co-microinjecting two independent transgenes encoding human APP$_{Swe}$ and the human tau$_{P301L}$ (both under control of the mouse Thy1.2 regulatory element) into single-cell embryos harvested from homozygous mutant PS1$_{M146V}$ knockin (PS1-KI) mice. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all appropriate measures were taken to minimize pain and discomfort in experimental animals.

Behavior

The T-maze consisted of a central main arm with two side arms positioned perpendicular to the main arm. The central arm was 65 cm long, and the two side arms were 30 cm each. The maze width was 13.5 cm. The walls of the maze were made of transparent acrylic and were 20 cm tall. At the beginning of each test, the mice were placed in the main stem while one side arm was blocked by a barrier so that the mice were forced to make a choice. Once the mice entered the side arm, the entrance was blocked, thus retaining them in the side arm. Mice were left to explore that arm for 120 seconds, at the end of which they were placed back in the main arm of the maze with both side arms open. Mice were free to choose the arm that they already explored or the new arm. Each animal was tested daily for 7 days, and on each day, we alternatively blocked one side arm. The numbers of alternations and the latency to make a choice during the free trial were recorded.

Immunological and Histological Staining

After completion of the behavioral tasks, the mice were transcardially perfused with ice-cold phosphate-buffered saline (PBS). After perfusion, each brain was cut sagit-tally and one half of the brain was frozen in dry ice, whereas the other half was fixed in ice-cold paraformaldehyde for 48 hours. After fixation, brains were cut (50 μm thick) using a slicing vibratome (Pelco, Redding, CA), and sections were stored in 0.02% sodium azide in PBS. Immunohistochemical analysis was conducted as previously described.[13] The number of plaques was quantified as previously described.[14]

Western Blot and Enzyme-Linked Immunosorbent Assay

The brains were homogenized in tissue protein extraction reagent (T-PER; Pierce, Rockford, IL) supplemented with a complete mini protease inhibitor tablet (Roche, Basel, Switzerland) and phosphatase inhibitors (Calbiochem, San Diego, CA). The homogenized mixes were briefly sonicated to sheer the DNA and centrifuged at 4°C for 1 hour at 100,000 $\times$ g. The supernatant was stored as the soluble fraction. The pellet was rehomogenized in 70% formic acid and centrifuged at 4°C for 1 hour at 100,000 $\times$ g. The supernatant was stored as the insoluble fraction. Protein concentration was determined using the Bio-Rad (Hercules, CA) protein assay, and samples were adjusted with T-PER to the same concentration. Western blot and $A\beta$ enzyme-linked immunosorbent assay experiments were done as previously described.[13,15]

Enzymatic Assays

GSK-3β and CDK5 activities were measured as previously described.[16]

Antibodies

The following antibodies were used in this study: anti-Aβ 6E10 (Signet Laboratories, Dedham, MA), anti-Aβ 1560 (Chemicon, Temecula, CA), anti-Aβ40 and anti Aβ42 (Biosource, Camarillo, CA), anti-Aβ 35–40 (MM32-13.1.1, for Aβ_{40}) or anti-Aβ 35–42 (MM40-21.3.4, for Aβ_{42}), anti-β-actin (Sigma, St. Louis, MO), anti-GSK-3, GSK-3β S9, GSK-3α S21, CDK5, phospho-c-Jun NH$_2$-terminal kinase (JNK) (Cell Signaling, Danvers, MA), anti-phospho cdc2 (Stressgen, Ann Arbor, MI), anti-tau HT7, (Innogenetics, Gent, Belgium), AT8, AT180, AT270, and AT100 (Pierce); 12E8 was a generous gift from Dr. Peter Seubert, Elan Pharmaceuticals, San Francisco, CA. HT7 recognizes tau independent of its phosphorylation state.

Statistical Analysis

Data were analyzed using one-way analyses of variance with Bonferroni post test using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA).
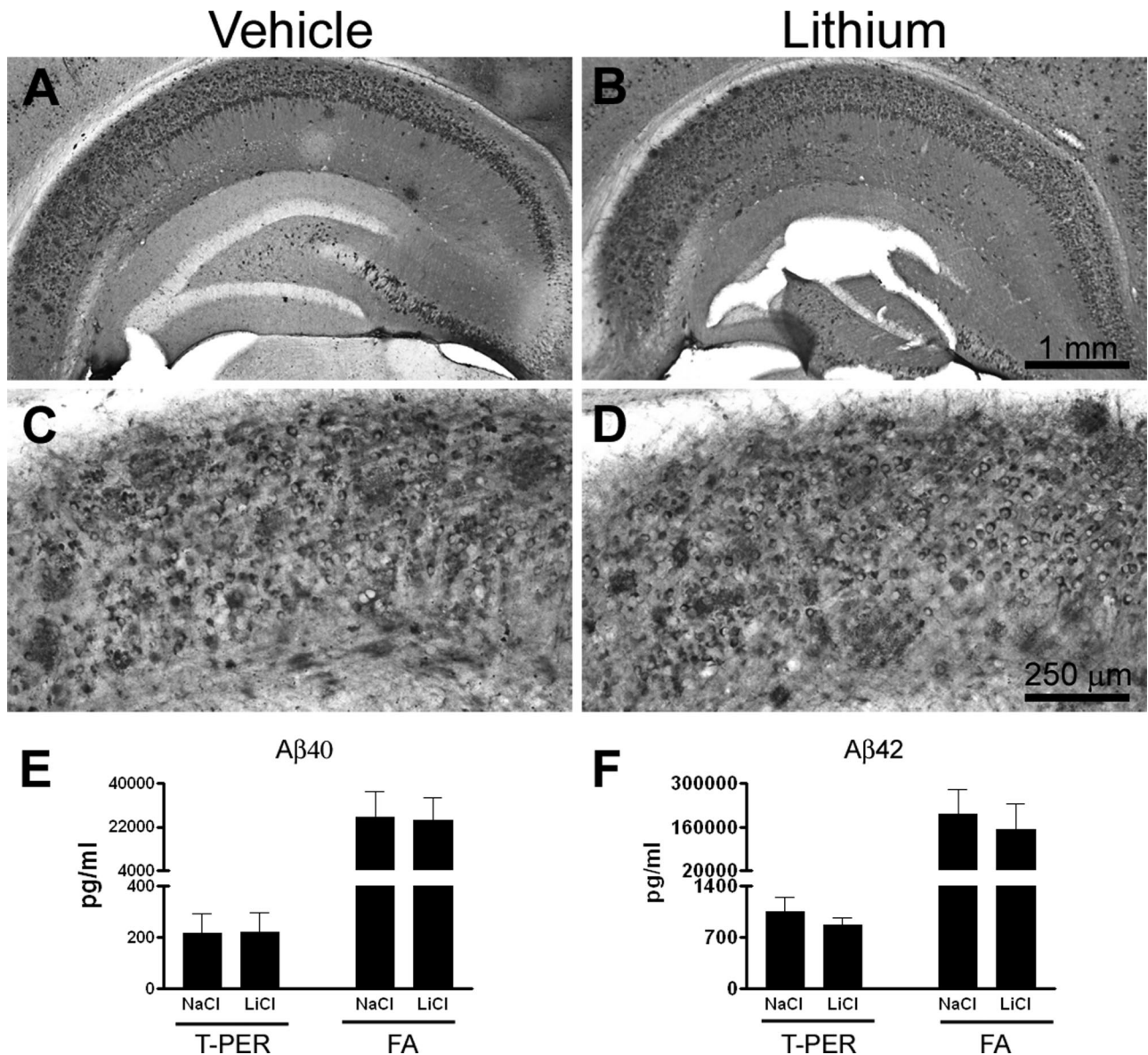
Vehicle   Lithium

Figure 1. The Aβ load is not altered by lithium treatment in 3xTg-AD mice with established plaque pathology. Low (**A**, **B**)- and high (**C**, **D**)-magnification photomicrographs of hippocampal brain sections from lithium-treated and untreated mice stained with the anti-Aβ antibody 6E10. No changes in the intraneuronal Aβ staining or plaque number were observed between treated and untreated mice ($n = 10$ per group). **E** and **F:** The steady-state levels of soluble and insoluble Aβ40 and Aβ42 were not significantly different between treated and untreated mice as determined by sandwich enzyme-linked immunosorbent assay ($n = 10$ per group). T-PER, tissue protein extraction reagent (Pierce); FA, formic acid.

Results

We chronically administered lithium (300 μl of 0.6 mol/L LiCl/mouse/day intraperitoneally) to 15-month-old homozygous 3xTg-AD and non-Tg mice ($n = 10$ per group) for a period of 4 weeks. Age- and gender-matched 3xTg-AD and non-Tg mice were injected with NaCl as a control. This treatment paradigm was based on previously published data.[5] In our hands, we found that this treatment regimen was well tolerated, and we did not observe any adverse effects, including any changes in the weight of the mice in any group during the treatment course (data not shown).

Lithium Does Not Alter the Aβ Load in 3xTg-AD Mice

Because reports suggest that lithium may reduce the Aβ burden in mice,[7,8] we first investigated whether it reduced the Aβ load in the 3xTg-AD mice by both immunohistochemical and biochemical measurements. Brain sections from lithium- and vehicle-treated mice were stained with several different antibodies against Aβ, including those specific for 1560, 4G8, 6E10, and Aβ42. At 15 months of age, the homozygous 3xTg-AD mice typically show advanced Aβ pathology, including intraneuronal Aβ and extracellular plaques, throughout the cor-
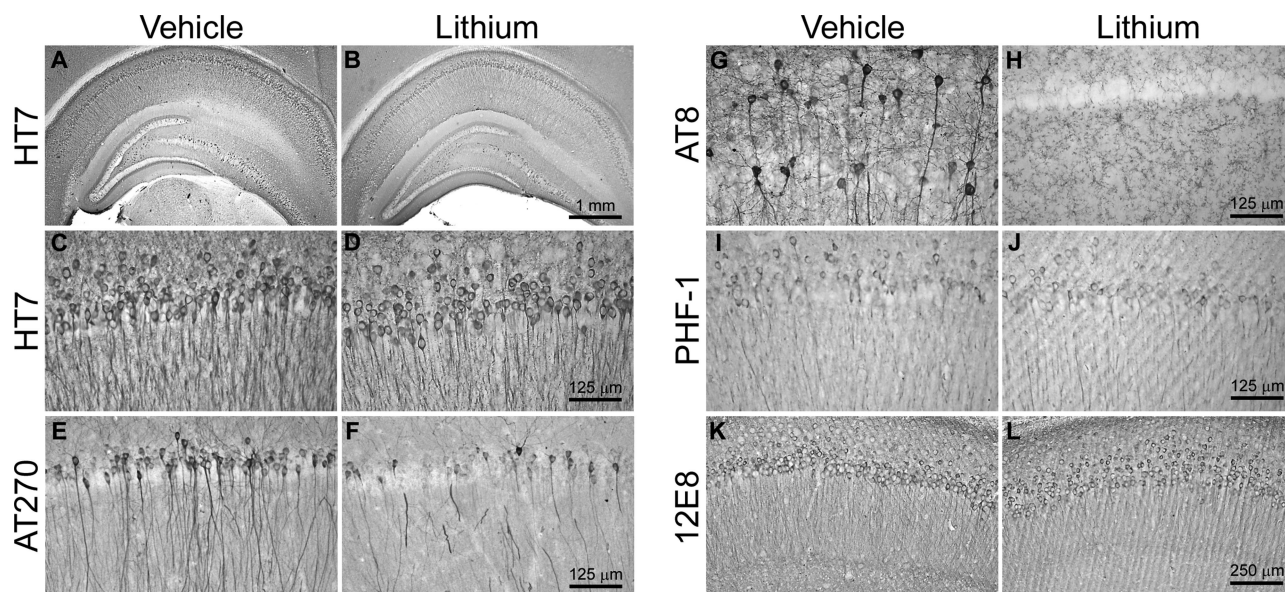
Figure 2. Reduced selective phosphotau epitopes in brains of lithium-treated mice. Representative microphotographs of sections from treated and untreated mice stained with different anti-tau antibodies. **A–D:** Low- and high-magnification views of the CA1 region of the hippocampus stained with antibody HT7 showing that lithium does not affect total tau levels. **E–H:** Lithium-treated mice showed a significant decrease in the somatodendritic accumulation of tau phosphorylated at Thr181 and Ser202/Thr205 compared with untreated mice in the CA1 region of the hippocampus. **I–L:** In contrast, lithium did not affect tau phosphorylation at the PHF-1 and 12E8 sites ($n = 10$ per group).

tex, hippocampus, and the amygdala. At the end of the treatment, we found no significant changes in intraneuronal Aβ or extracellular Aβ deposits in the brains of treated versus untreated mice (Figure 1, A–D). The number of plaques per microscopic field was 25 ± 3.8 SEM and 27 ± 3.1 SEM for the lithium- and control-treated mice, respectively.

To quantitatively assess the effect on Aβ levels, we next assayed brain extracts by sandwich enzyme-linked immunosorbent assay and found that lithium did not significantly alter the steady-state levels of Aβ40 or Aβ42 in either the soluble and insoluble fractions (Figure 1, E and F). These data differ from another study in which lithium was found to reduce Aβ pathology.[7,8] One notable disparity between our study and that of Phiel and colleagues[7] relates to the stage of the Aβ pathology at the time the treatment was initiated; although the mice were treated for the same time period, in the former study, treatment was commenced before thioflavine-positive fibrillar plaques were observed, whereas we began treatment after these lesions developed in the 3xTg-AD mice. Likewise, Su and colleagues[8] found that prolonged lithium treatment in young mice prevented the development of plaques. Taken together, the sum of these studies suggest that lithium may be useful for lowering the Aβ load during early stages of AD but not once advanced plaques have formed.

Lithium Administration Selectively Reduces Tau Phosphorylation

To determine the efficacy of lithium on the tau pathology, we immunohistochemically evaluated sections from lithium- and saline-treated 3xTg-AD mice with different anti-tau antibodies (Figure 2). By 15 months of age, the homozygous 3xTg-AD mice show extensive tau immunoreactivity in the somatodendritic compartment of CA1 pyramidal neurons, in the cortex and amygdala.[10,11,14] Lithium did not alter somatodendritic tau levels, as detected by the total tau antibody HT7, which were similar between treated and untreated mice (Figure 2, A–D). Number of pixels per microscopic field was $14,565 \pm 4234$ SEM and $13,956 \pm 5021$ SEM for the treated and untreated mice, respectively. In contrast, we found that several phosphotau epitopes were selectively reduced in the lithium-treated mice, including tau phosphorylated at Thr181 (as detected by antibody AT270) and Ser202/Thr205 (as detected by antibody AT8) (Figure 2, E–H). As expected, phosphorylation at other sites, including the 12E8 site, which is phosphorylated by microtubule affinity-regulating kinase,[17] was unaltered by lithium treatment, as was phosphorylation at the PHF-1 site, which represents a late stage marker in the 3xTg-AD mice (Figure 2, I–L).

To evaluate quantitatively the effects of lithium on tau pathology, we next analyzed the changes in the steady-state levels of tau and its phosphorylation pattern by Western blot. We first observed that total steady-state levels of tau were comparable between treated and untreated mice (Figure 3, A and B). In contrast, we observed a marked reduction in the steady-state levels of phosphotau recognized by AT270, AT8, AT180, and AT100 (Figure 3, A, and C–F). This finding is consistent with previous work showing that these sites are phosphorylated by GSK-3β.[3] Conversely, the steady-state levels of phosphotau recognized by PHF-1 or 12E8 were comparable in the brains of treated versus untreated mice (Figure 3, A, G, and H). Although phosphorylation at the PHF-1 site may also be mediated by GSK-3β, CDK5
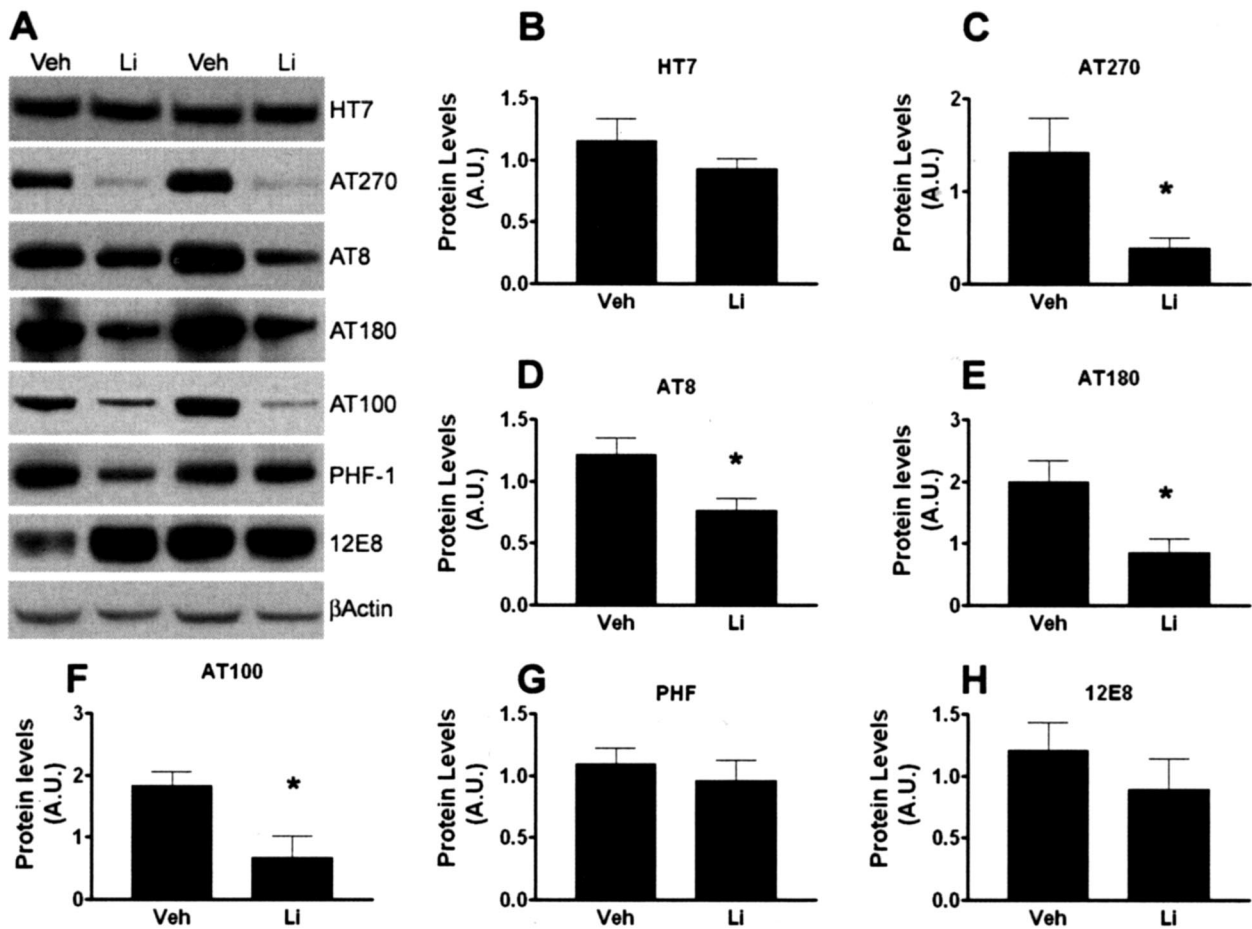
Figure 3. Lithium selectively lowers steady-state levels of certain phosphotau epitopes. **A:** Protein extract from brains of treated and untreated mice were separated in a Western blot probed with different anti-tau antibodies ($n = 10$ per group). Representative Western blots are shown. **B–H:** Densitometric analysis of the Western blots showed that the steady-state levels of tau, as detected by antibody HT7, were not significantly different between treated and untreated mice (**B**), whereas there was a significant decrease in the steady-state levels of AT270 ($P = 0.037$, **C**), AT8 ($P = 0.011$, **D**), AT180 ($P = 0.031$, **E**), and AT100 ($P = 0.033$, **F**). Lithium, however, did not significantly change the steady-state levels of PHF-1 and 12E8 (**G, H**). Veh, vehicle; Li, lithium; A.U., arbitrary units.

has also been implicated in the phosphorylation of this site.[3,18–21]

To elucidate the mechanism underlying the reduction in tau phosphorylation in the lithium-treated mice, we used quantitative Western blot analysis to measure the levels of GSK-3α and GSK-3β. We found that lithium did not significantly change the steady-state levels of these two kinases (Figure 4, A and B), which is consistent with previous work.[5] It has been shown that phosphorylation at Ser21 inhibits GSK-3α activity, whereas phosphorylation at Ser9 inhibits GSK-3β activity.[22] We measured the levels of GSK-3α phosphorylated at Ser21 and GSK-3β phosphorylated at Ser9 and found that the inactive forms of both GSK-3α and GSK-3β were increased in the lithium-treated mice (Figure 4, A, C, and D). We next directly measured the enzymatic activity of these two enzymes. Compared with vehicle-treated mice, the enzymatic activity of GSK-3α and GSK-3β was significantly lower in the brains of lithium-treated mice (Figure 4, E and F). Another major kinase involved in tau phosphorylation is CDK5,[2] which seems unaffected by lithium. We measured the steady-state levels of this kinase and found that its activity was unaltered

between treated and untreated mice (Figure 4, A and G). In addition, we found that lithium treatment did not change the levels of p35 and p25, two activators of CDK5 (Figure 4, A and H). Other kinases, such as cdc2, JNK, and MAP kinase, have also been involved in tau phosphorylation. Using antibodies against the activated forms of these enzymes, we found that lithium does not change their activity (Figure 4, A, and I–K). These data suggest that the decrease in tau phosphorylation induced by lithium treatment is mediated by the selective reduction of GSK-3α and GSK-3β activity.

Lithium Does Not Rescue Working Memory Deficits

We next tested treated and untreated mice in the T-maze, which relies on the tendency of mice to freely alternate choices during successive trials; this paradigm is considered a working memory task and is dependent on several brain regions including the basal forebrain, hippocampus, and prefrontal cortex.[23] Whereas control-treated non-Tg mice alternated 72.15 ± 2.4% of the time
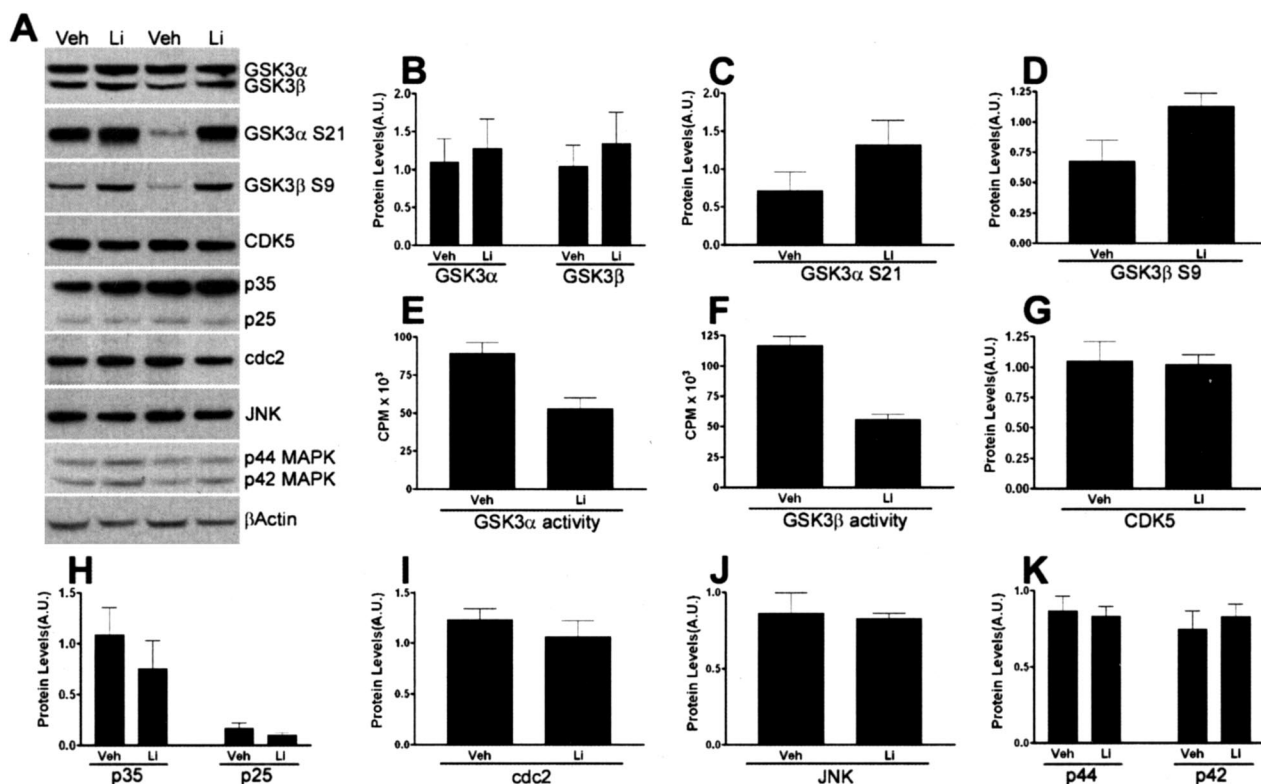
Figure 4. Lithium lowers GSK-3α and GSK-3β activity in the brains of the 3xTg-AD mice. **A:** Protein extract from brains of treated and untreated mice were separated in Western blots probed with different antibodies to determine the activity of GSK-3α, GSK-3β, CDK5, cdc2, JNK, and MAP kinase ($n = 10$ per group). **B:** Densitometric analysis of the Western blots showed that the steady-state levels of total GSK-3α and GSK-3β were similar between treated and untreated mice ($P > 0.05$). **C** and **D:** In contrast, the levels of the inactive forms of GSK-3α and GSK-3β were significantly increased in the lithium-treated mice compared with vehicle-treated mice ($P < 0.05$). **E** and **F:** To assess the changes in GSK-3 activity, we directly measured the enzymatic activity of these two kinases and found that lithium administration significantly decreased the activity of GSK-3α and GSK-3β ($P = 0.002$ and $P < 0.0001$, respectively). **G–K:** The effects of lithium were selective for GSK-3 because we found that the levels of CDK5 or its activators p35 and p25, cdc2, JNK, and p44/42 MAP kinase were not significantly different between treated and untreated mice. Veh, vehicle; Li, lithium; A.U., arbitrary units.

during the trials, vehicle-treated 3xTg-AD mice failed to alternate between the two arms of the maze on successive trials and performed at chance levels ($55.21 \pm 5.8\%$, Figure 5). Likewise, lithium did not significantly improve the performance of 3xTg-AD mice in this task, nor was the performance of non-Tg significantly improved ($55.71 \pm 5.6\%$ and $66.67 \pm 8.7\%$, respectively; Figure 5). As the ability to alternate successfully with each successive trial is considered to reflect intact working memory,[23] these results indicate that 1 month of lithium treatment is unable
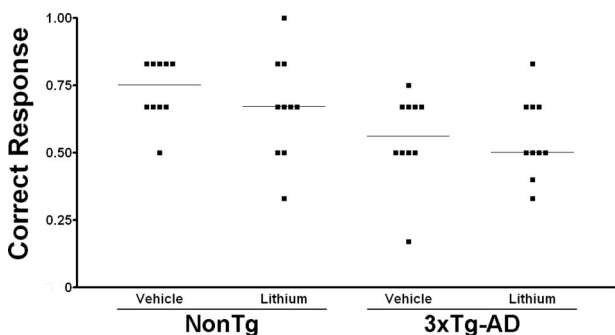

Figure 5. Lithium failed to rescue working memory deficits in the 3xTg-AD mice. Lithium treatment did not rescue the working memory deficits present in aged 3xTg-AD mice because no statistically significant difference was detected between the alternation rate of treated and untreated mice ($n = 10$ per group).

to rescue deficits in working memory in 16-month-old 3xTg-AD mice, despite the reduction in selective phosphotau epitopes.

Discussion

In this study, we administered lithium to 15-month-old 3xTg-AD mice daily for 1 month. Although this treatment paradigm had no effect on the Aβ pathology, we found that it significantly decreased tau phosphorylation at selective sites. Although several reports previously showed that lithium can successfully lower tau phosphorylation, and here we describe similar results in the 3xTg-AD mice, to our knowledge, no reports have yet investigated whether lithium is capable of rescuing the cognitive deficits. Here, we found that lithium administration did not rescue the working memory deficits in the 3xTg-AD mice. We have previously shown that reduction of soluble Aβ and soluble tau ameliorates the cognitive decline in the 3xTg-AD mice, whereas reduction of soluble Aβ alone did not.[24] These data, together with those presented here, indicate that a decrease in both Aβ and tau pathology is necessary to rescue the cognitive decline in an animal model with established plaques and tangles.

The reduction in tau phosphorylation was selective because we found a reduction in phosphotau only at

GSK-3β-targeted residues, such as Thr181 (detected by antibody AT270), Ser202;Thr205 (detected by antibody AT8), Thr231 (detected by antibody AT180), and Thr212; Ser214 (detected by antibody AT100). We did not find changes in tau phosphorylation at Ser396/404 (detected by antibody PHF-1). This site seems to be phosphorylated by GSK-3β[3] as well as CDK5[18–21]; therefore, it is likely that a combined inhibition of both kinases may be necessary to reduce phosphorylation of the PHF-1 site. In the 3xTg-AD mice, we have noted that AT270, AT180, and AT100 represent early markers of tau phosphorylation, whereas reactivity with AT8 and PHF-1 represents mid and late stages. Therefore, this treatment reduced tau phosphorylation at the early and mid stage but not at a late marker such as PHF-1.

Although the effects of lithium on Aβ production *in vitro* are controversial,[7,9] there is evidence showing that lithium may have a beneficial effect on preventing Aβ pathology *in vivo*. In particular, it has been shown that lithium reduces Aβ levels in APP/PS1 double-transgenic mice[7]; however, the effect of this treatment on plaques was not reported. In another study, it has been shown that administration of lithium to prepathological mice for 6 months prevented Aβ deposition in single APP transgenic mice.[8] In this study, we determined whether lithium could reverse established Aβ pathology or lower Aβ levels in aged mice and found that, at least with the experimental paradigm used here, lithium administration did not change Aβ load. Taken together, these data suggest that lithium alone may be a useful agent during early stages of AD, but not once extensive amyloid plaques have built up in the brain. However, it is possible that a longer lithium treatment may reduce plaque load even in mice with advanced plaque pathology.

The data presented here are consistent with previous work showing that lithium reduces tau phosphorylation *in vivo*[5] and further suggest that lithium may also be useful for treating tauopathies other than AD. For AD, the combination of lithium with other Aβ-mediated therapies may be advantageous. For example, we previously showed that Aβ immunotherapy can reduce early but not late phosphotau sites in the 3xTg-AD mice, whereas phosphorylation at the AT8 site was not reduced by Aβ immunotherapy[13]; here, we find that phosphorylation at this site is reduced by lithium treatment. Therefore, combining anti-Aβ interventions with lithium may offer the greatest beneficial effects for treating AD.

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